



# The effects of ionizing radiation on the structure and antioxidative and metal binding capacity of the cell wall of microalga *Chlorella sorokiniana*

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1 **The effects of ionizing radiation on the structure and antioxidative and metal-**  
2 **binding capacity of the cell wall of microalga *Chlorella sorokiniana***

3

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23

24 **Abstract**

25 The impact of ionizing radiation on microorganisms such as microalgae is a topic of  
26 increasing importance for understanding the dynamics of aquatic ecosystems in  
27 response to environmental radiation, and for the development of efficient approaches  
28 for bioremediation of mining and nuclear power plants wastewaters. Currently, nothing  
29 is known about the effects of ionizing radiation on the microalgal cell wall, which  
30 represents the first line of defence against chemical and physical environmental  
31 stresses. Using various microscopy, spectroscopy and biochemical techniques we show  
32 that the unicellular alga *Chlorella sorokiniana* elicits a fast response to ionizing radiation.  
33 Within one day after irradiation with doses of 1 to 5 Gy, the fibrillar layer of the cell wall  
34 became thicker, the fraction of uronic acids was higher, and the capacity to remove the  
35 main reactive product of water radiolysis increased. In addition, the isolated cell wall  
36 fraction showed significant binding capacity for  $\text{Cu}^{2+}$ ,  $\text{Mn}^{2+}$ , and  $\text{Cr}^{3+}$ . The irradiation  
37 further increased the binding capacity for  $\text{Cu}^{2+}$ , which appears to be mainly bound to  
38 glucosamine moieties within a chitosan-like polymer in the outer rigid layer of the wall.  
39 These results imply that the cell wall represents a dynamic structure that is involved in  
40 the protective response of microalgae to ionizing radiation. It appears that microalgae  
41 may exhibit a significant control of metal mobility in aquatic ecosystems via biosorption  
42 by the cell wall matrix.

43

44 **Keywords:** Alga; Cell wall; Copper; Radiation

45

## 46 **1. Introduction**

47 Microalgae are exposed to variable doses of ionizing radiation from natural (e.g.  
48 radionuclides from soil and rocks), and anthropogenic sources (radioactive waste,  
49 radionuclides from mining/ores, nuclear power plant accidents and nuclear testing)  
50 (UNSCEAR, 2011). Because photosynthetic microalgae are primary producers of  
51 biomass and oxygen, any damaging effects of radiation on these microorganisms will  
52 directly impact the function and organization of aquatic ecosystems (Fuma et al., 2012;  
53 Nascimento and Bradshaw, 2016). Conversely, microalgae appear to show resilience to  
54 radiation stress; for example they are very efficient in the remediation of freshwaters  
55 that are contaminated with radioactive metals, such as strontium, uranium, and caesium  
56 (Fukuda et al., 2014; Kalin et al., 2005; Vanhoudt et al., 2018). Furthermore, some  
57 microalgal species are known to colonize spent nuclear fuel storage pools and uranium  
58 tailings ponds showing high levels of radiation and heavy metals pollution (Baselga-  
59 Cervera et al., 2018; McGraw et al., 2018; Rivasseau et al., 2016). Seasonal algal  
60 blooms in the spent nuclear fuel storage at Sellafield, UK, is a phenomenon that  
61 probably best illustrates the potential of microalgae to adapt to radiation and to thrive in  
62 such ecosystems (Foster et al., 2020; McGraw et al., 2018). It is worth mentioning that  
63 these properties demonstrate that microalgae are capable pioneer species in the  
64 colonization of highly inhospitable environments (Baselga-Cervera et al., 2018;  
65 Rivasseau et al., 2016). Clearly, the effects of ionizing radiation on microalgae and the  
66 mechanisms of their adaptation are of fundamental environmental interest, as  
67 highlighted recently by the disastrous contamination of water in the Fukushima-Daiichi  
68 nuclear power plant accident (Fukuda et al., 2014). It is important to note that high

69 energy electromagnetic radiation (such as gamma and X) is the most relevant ionizing  
70 radiation in aquatic systems. It has high penetrating power, and energy that is sufficient  
71 to cause radiolysis of water and to directly oxidize/damage biomolecules (LaVerne,  
72 2000).

73 It is known that the exposure of microalgae to radiation may result in oxidative damage  
74 of lipids and DNA and decreased photosynthetic efficiency, growth and survival  
75 (Boreham et al., 1993; Gomes et al., 2017; Rea et al., 2008), as well as in upregulated  
76 antioxidative defence and photoprotection, alterations in carbohydrate and general  
77 metabolic profile, and other traits of radioresistance (Bradshaw et al., 2019; Foster et  
78 al., 2020; Golz and Bradshaw, 2019; McGraw et al., 2018; Santier et al., 1985).  
79 However, the response at the level of the cell wall has not yet been examined. The cell  
80 wall is the zone of contact between the microalgal cell and the environment, and the first  
81 line of chemical and physical defence (Baudelet et al., 2017). It represents a dynamic  
82 multi-layer structure that is actively involved in the adaptation to different stressors  
83 (Beacham et al., 2014; Jeong et al., 2017; Yap et al., 2016). Pertinent to this, the cell  
84 wall is the main (radio)metal sequestering (*i.e.* biosorbent) component of microalgal  
85 biomass (Hadjoudja et al., 2010; Horikoshi et al., 1979; Vanhoudt et al., 2018). Finally,  
86 the thickness and structure of the microalgal cell wall is of technological relevance as it  
87 represents a key biological parameter for efficient lipid extraction during microalgal  
88 biodiesel production (Anto et al., 2020; Yap et al., 2016). Additionally it is of great  
89 relevance as a source of carbohydrates for microalgal bioethanol production  
90 (Hernández et al., 2015).

91 The aim of our study was to determine the effects of ionizing radiation (X-rays) on the  
92 cell wall of *Chlorella sorokiniana*. We analyzed: (i) the structure of the cell wall using  
93 transmission electron microscopy (TEM) and Fourier-transform infra-red spectroscopy  
94 (FTIR); (ii) the capacity of cell wall isolates to remove hydroxyl radical (HO·) using  
95 electron paramagnetic resonance (EPR) spin-trapping spectroscopy; and (iii) the  
96 capacity of cell wall isolates to bind heavy metals using biochemical assays and EPR.  
97 *C. sorokiniana* was selected as a widely used model microalga with high potential for  
98 application in industry and wastewater treatment (Lizzul et al., 2018), as it is also  
99 commonly found in many freshwater ecosystems.

## 100 **2. Material and methods**

### 101 2.1. Cell cultivation

102 *C. sorokiniana* (strain CCAP 211/8K; alternative designation UTEX 1230) was obtained  
103 from the Culture Collection of Algae and Protozoa, UK. Algal inocula were added to 3N-  
104 BBM+V medium; 35 mL in 50 mL Erlenmeyer flasks (TEM experiments), or 150 mL in  
105 250 mL flasks (all other experiments). Initial density in all samples was  $5 \times 10^5$  cells/mL.  
106 Algae were grown for 20 days at 22°C on an orbital shaker (120 rpm) in growth cabinet  
107 with a continuous photon flux of  $120 \mu\text{mol m}^{-2} \text{s}^{-1}$  (MST TL-D Reflex 36W840 1 SLV/25  
108 tubes, Philips, Amsterdam, Netherlands). At day 20, cultures were in the stationary  
109 phase, as determined by optical density ( $\text{OD}_{750}$  was  $7.2 \pm 0.5$ ) and biomass ( $2.0 \pm 0.2$   
110 mg/mL). The volume of samples was corrected for evaporation at day 20 with sterile  
111 deionized water. Samples were irradiated and returned to the growth cabinet for  
112 additional 24 h, and then microalgae were collected for further analysis or processing.

### 113 2.2. Irradiation protocol

114 Aliquots of culture (35 mL) were placed in a Petri dish and exposed to X-ray irradiation  
115 using CellRad irradiation chamber (Faxitron Bioptics LLC, Tucson, AZ, USA; tube  
116 power: 750 W; focal spot size: 1.0 x 1.4 mm; filtration: 1.6 mm Be and 0.5 mm Al; beam  
117 angle: 40° divergence; dosimeter: ion chamber). Energy was kept constant at 120 kV;  
118 doses and rates were adjusted by changing the current (the doses released by the  
119 source were 10, 20 and 50 Gy). Total absorbed doses were 1.09 Gy (rate, 0.25 Gy/min;  
120 exposure time 4.4 min), 2.21 Gy (rate, 0.25 Gy/min; 8.9 min), and 5.45 Gy (rate, 0.55  
121 Gy/min; 9.9 min). For simplicity, we refer to these absorbed doses as 1, 2, and 5 Gy.  
122 The effects of acute irradiation on cell viability were tested using Evans Blue stain  
123 (Sigma-Aldrich, St. Louis, MI, USA), as described previously (Zuppini et al., 2007).  
124 Evans Blue is a commonly used measure of cell viability; non-viable cells loose cellular  
125 permeability allowing accumulation of the Evans Blue dye, therefore an increased  
126 proportion of Evans Blue stained cells in a population indicates an increased proportion  
127 of non-viable cells. The viability is presented as a percentage of Evans Blue negative  
128 cells. At least 100 cells were analysed per sample.

### 129 2.3. TEM microscopy

130 Cells were spun down at 5000 g for 5 min, and fixed overnight at 4°C in 0.1 M  
131 phosphate buffer (PB; pH 7.2) containing 3% (v/v) glutaraldehyde and 1% (v/v)  
132 paraformaldehyde (Serva, Heidelberg, Germany). Post-fixation was performed with  
133 1% (w/v) osmium tetroxide (Serva) in PB for 2 h at room temperature. Samples were  
134 dehydrated in a graded acetone series and then embedded in resin for soft blocks  
135 (AGR1031, Agar Scientific, Stansted, UK). Ultra-thin sections (70 µm), obtained with a  
136 Leica UC7 ultramicrotome (Leica Microsystems, Wetzlar, Germany), were stained for 15

137 minutes with 1% uranyl acetate and 5 minutes with 3% lead citrate and observed at 60  
138 kV in a JEOL JEM-1010 TEM (Jeol, Tokyo, Japan). The thickness of cell wall layers  
139 was measured using ImageJ (NIH). At least 25 cells with the nuclear mid-section from  
140 the control and each irradiation dose treatment were analysed. The thickness of cell  
141 wall layers was measured at four points (on the x and y axis of the micrograph with 0  
142 point at the cell's centre).

#### 143 2.4. Cell wall isolation

144 Biomass from 150 mL samples (untreated and irradiated with different doses) was spun  
145 down and washed 2× in 5 mL distilled water by centrifugation at 2300 g / 5 min. Cell wall  
146 isolation was conducted according to the previously described protocol with slight  
147 modification (Simonović et al., 2011). Protocol steps were performed at room  
148 temperature if not indicated otherwise: (i) homogenization in mortar with liquid N<sub>2</sub> and  
149 collection of the sample (~0.5 g fresh weight) with 5 mL water; (ii) 2× wash with 5 mL  
150 water by centrifugation at 800 g / 5 min; (ii) 10× shake (15 min) and wash (800 g / 5  
151 min) with 5 mL of chloroform:methanol (1/1 v/v) mixture; (iii) Overnight incubation in the  
152 chloroform:methanol solution at 4°C; (iv) shake (1 h) and wash (800 g / 5 min) in 5 mL 1  
153 M NaCl; (iv) shake (1 h) in 5 mL 0.5% Triton and 5× wash (800 g / 5 min) with 5 mL  
154 water; (v) shake (20 min) and wash (800 g / 5 min) in 5 mL methanol; (vi) 4× shake (20  
155 min) and wash (800 g / 5 min) in 5 mL acetone; (vii) dry overnight at 30°C; (viii) 2×  
156 incubation with 5000 U of amylase (Megazyme, Wicklow, Ireland) per 1 g of sample in  
157 PB (pH 7.2) for 24 h at 30°C to remove starch. (ix) wash with 5 mL PB several times  
158 until supernatant becomes clear; (x) 2× shake (20 min) and wash (800 g / 5 min) with 5  
159 mL acetone, and leave to dry. The protocol was aimed at preserving the structure of all



160 cell wall polymers (Chen et al., 2000; Hall and Moore, 1983). Gravimetry of isolated cell  
161 wall and fresh weight of samples was performed. The composition of the isolated cell  
162 wall was analysed by mid-infrared FTIR (4000-400  $\text{cm}^{-1}$ ) using a Nicolet 6700  
163 spectrometer (Thermo Scientific, Waltham, MA, USA). The resolution of spectra was 1  
164  $\text{cm}^{-1}$ .

## 165 2.5. Metal-binding capacity

166 Cell wall isolates were tested for capacity to bind  $\text{Cu}^{2+}$ ,  $\text{Mn}^{2+}$ , and  $\text{Cr}^{3+}$  ions. Isolates (0.5  
167 mg) were placed into 2 mL of 5 mM solutions of  $\text{CuCl}_2$ ,  $\text{MnCl}_2$ , or  $\text{KCr}(\text{SO}_4)_2$  (Sigma-  
168 Aldrich), that were prepared in 20 mM Hepes buffer (pH 7.5). Samples were vigorously  
169 shaken for 10 min in polypropylene tubes (Eppendorf, Hamburg, Germany), and  
170 centrifuged at 13000 g / 10 min. Supernatant was collected for biochemical assays,  
171 whereas pellet was collected for EPR spectroscopy. The concentration of  $\text{Cu}^{2+}$  in the  
172 supernatant was determined using the fluorescent probe Fura-2 (Sigma-Aldrich), as  
173 described previously (McCall and Fierke, 2000), on a FluoroLog 3 fluorimeter (Horiba,  
174 Kyoto, Japan) with excitation at 340 nm and emission at 510 nm. Concentrations of two  
175 other metals were determined according to previously described colorimetric assays:  
176  $\text{Mn}^{2+}$  with formaldoxime reagent at  $\lambda = 450$  nm (Goto et al., 1962), and  $\text{Cr}^{3+}$  with xylenol  
177 orange at  $\lambda = 530$  nm (Cheng, 1967). Working solutions for calibration curves were  
178 prepared daily by stepwise dilution from standard stock solution. Samples were diluted  
179 prior to measurements to match the calibration range. The decrease in the  
180 concentration of metals in the buffer was used to calculate the binding capacity ( $\mu\text{g}$  of  
181 bound metal per mg of cell wall isolate).

## 182 2.6. Antioxidative capacity of the cell wall

183 The capacity of isolated cell wall (0.5 mg in 50  $\mu$ L water sample) to scavenge HO $\cdot$   
184 radical was established using the Fenton reaction, a HO $\cdot$ -generating system: Fe $^{2+}$  (1  
185 mM; Sigma-Aldrich) + H $_2$ O $_2$  (3 mM; Carlo Erba Reagents, Milano, Italy), and an EPR  
186 spin-trapping method with DEPMPO spin-trap (5-diethoxyphosphoryl-5-methyl-1-  
187 pyrroline-N-oxide; Santa Cruz Biotechnologies, Dallas, TX, USA) at the final  
188 concentration of 5 mM. Deionized ultrapure 18 M $\Omega$  water was used in all experiments.  
189 The pH of samples was  $\sim$ 6.5. Spectra were recorded after 2 min incubation at room  
190 temperature, using Bruker EMX Nano X-band (9.65 GHz) spectrometer with the  
191 following settings: power attenuation, 25 dB; modulation amplitude, 0.2 mT; modulation  
192 frequency, 100 kHz; sweep time, 2 min. Antioxidative capacity to remove HO $\cdot$  was  
193 calculated using the amplitude (A) of DEPMPO/OH signals according to the following  
194 equation:  $(A_{\text{Fenton}} - A_{\text{Fenton} + \text{cell wall}})/A_{\text{Fenton}}$  (Spasojević et al., 2009).

## 195 2.7. EPR spectroscopy of transition metals

196 Collected pellets (cell wall isolates with bound metals) were placed into 100  $\mu$ L of Hepes  
197 buffer. Samples were vortexed, placed into quartz tubes, and snap-frozen in liquid N $_2$ .  
198 Measurements were performed at 77 K on a Bruker EMX Nano spectrometer with finger  
199 dewar with liquid N $_2$ , using the following settings: power attenuation, 25 dB; modulation  
200 amplitude, 0.8 mT; modulation frequency, 100 kHz; sweep time, 1 min; number of  
201 scans/accumulations, 10. Spectra of Cu $^{2+}$ , Mn $^{2+}$  and Cr $^{3+}$  (1.5 mM) in Hepes buffer were  
202 recorded for comparison as blanks. Spectrum of Cu $^{2+}$  (1.5 mM) in the presence of  
203 chitosan (10 mM) was recorded to analyse interactions with cell wall. Spectral  
204 simulations were performed to establish  $g$ -values and hyperfine splitting (A), using  
205 Hyperfine Spectrum Software (WF Hagen, TU Delft, The Netherlands) (Hagen, 2008).

## 206 2.8. Statistics

207 All experiments were performed in three biological replicates, except for the viability test  
208 (four replicates) and cell wall isolation (five replicates). Results are presented as mean  $\pm$   
209 standard error (SE) where appropriate. Statistical significance ( $p < 0.05$ ) in comparison  
210 to control experiments was calculated using Mann-Whitney U test.

## 211 3. Results

212 TEM micrographs illustrate the structure of the *C. sorokiniana* cell wall, which is  
213 composed of a trilaminar sheath (TLS) and fibrillar wall (Fig. 1a). TLS appears as a  
214 translucent line inserted between two electron dense lines; the outer layer (mature  
215 mother wall) is separated by an electron translucent space from the thin inner layer  
216 (daughter wall). The fibrillar wall is located between the TLS and plasma membrane  
217 (Baudeflet et al., 2017). The established thickness of the cell wall in untreated *C.*  
218 *sorokiniana* is in accordance with data published in the past (Martinez et al., 1991).  
219 Irradiation did not induce significant changes in the TLS. On the other hand, the  
220 thickness of the fibrillar wall was significantly increased one day after irradiation with 1  
221 and 2 Gy (Fig. 1b). In line with this observation, the yield of cell wall was increased by  
222 ~50% in irradiated microalgae (Fig. 1c). The slight change in the fibrillar wall following 5  
223 Gy of irradiation was not statistically significant. It is noteworthy that there were no  
224 significant changes to cell viability in response to irradiation:  $90.8 \pm 0.7\%$  in controls;  
225  $84.1 \pm 4.1\%$  for algae exposed to 1 Gy;  $74.7 \pm 1.4\%$  for 2 Gy; and  $75.4 \pm 1.1\%$  for 5 Gy.  
226 Cell wall thickness is an important biological and technological parameter. It provides  
227 chemical (including antioxidative) and mechanical protection from the surroundings,  
228 determines the carbon budget of the cell, and affects the extractability of lipids and

229 pigments as well as other industrially-relevant properties of microalgal biomass (Anto et  
230 al., 2020; Baudelet et al., 2017; Jeong et al., 2017). The thickening of the cell wall  
231 appears to be a common response of microalgae to other types of environmental stress,  
232 such as N-deficiency and changes in salinity (in marine algae), under different growing  
233 conditions (Beacham et al., 2014; Jeong et al., 2017; Yap et al., 2016). In accord with  
234 our findings, the exposure of *Nannochloropsis* to N-stress has been reported to result in  
235 the thickening of the inner cellulose-based sheath of a bilayer cell wall (Jeong et al.,  
236 2017).

237 We applied FTIR spectroscopy to analyze the effects of radiation on cell wall  
238 composition. FTIR spectra of cell wall isolates showed strong absorption peaks at 3290,  
239 2940, 1645, 1535, 1446, 1385, 1237, 1147, and 1055  $\text{cm}^{-1}$  (Fig. 2). The assignment was  
240 performed according to available FTIR spectra of microalgal biomass, which mainly  
241 reflect the functional groups in the cell wall (Dmytryk et al., 2014; Driver et al., 2015;  
242 Hadjoudja et al., 2010; Petrovič and Simonič, 2016). The cell wall composition of *C.*  
243 *sorokiniana* is not fully known. However, available data imply that *Chlorella* cell wall is  
244 composed of: (i) a “rigid wall” (sheaths in TLS), which contains glucosamine and N-  
245 acetylglucosamine in the form of a chitosan-like polymer; and (ii) a plastic polymeric  
246 matrix (fibrillar wall), which is composed of rhamnose, galactose, uronic acids  
247 (glucuronic acid in *C. sorokiniana*), arabinose, mannose, and other sugars (Baudelet et  
248 al., 2017; Russell, 1995). The band at 3290  $\text{cm}^{-1}$  was assigned to glucosamine (N-H  
249 stretching), and neutral sugars (O-H and C-O stretching). Bands at 1645, 1535 and  
250 1055  $\text{cm}^{-1}$  mainly come from chitosan-like polysaccharides. The 1055  $\text{cm}^{-1}$  band was  
251 weaker in irradiated samples, which may come from oxidation-induced breakage of

252 polymeric chains (Wasikiewicz et al., 2005). On the other hand, the bands derived from  
253 carboxyl and carbonyl groups were stronger in cell wall isolated from irradiated  
254 microalgae. This implies that *C. sorokiniana* accumulates uronic acids in the fibrillar wall  
255 in response to ionizing radiation. It is noteworthy that no bands corresponding to either  
256 phosphoryl or sulfone groups were detected, which is in line with available data on cell  
257 wall composition in *Chlorella* (Baudelet et al., 2017). Next we examined the impact of  
258 irradiation on the capacity of the cell wall to scavenge HO<sup>·</sup>, the main oxidizing species  
259 produced in water radiolysis (LaVerne, 2000). The exposure of *C. sorokiniana* to  
260 radiation led to significant increase of antioxidative capacity of the cell wall (Fig. 3). Of  
261 note, less intensive spectra stand for more antioxidative capacity. It has been proposed  
262 previously that plants 'rely' on non-enzymatic antioxidative defence against HO<sup>·</sup>, such  
263 as carbohydrates and cell wall polymers (Bogdanović Pristov et al., 2011; Spasojević et  
264 al., 2009). There is no enzymatic system for the removal of this radical. The increased  
265 antioxidative capacity could be explained by a higher fraction of uronic acids in the cell  
266 wall of irradiated microalgae. A number of studies have found that uronic acid-rich  
267 macromolecules, such as xylan (glucuronic acid) and pectin (galacturonic acid), are  
268 highly susceptible to reactions with radicals, including HO<sup>·</sup> (Akhlaq et al., 1990; Fry,  
269 1998; Zegota, 2002). We have shown in a comparative study of antioxidative activities  
270 of plant cell wall components that pectin and xylan are the most efficient HO<sup>·</sup>  
271 scavengers (Bogdanović Pristov et al., 2011). Alternatively, higher antioxidative capacity  
272 may be related to radiation-induced fragmentation of chitosan-like polymer. Several  
273 studies have reported that such fragmentation results in improved antioxidative  
274 performance (Abd El-Rehim et al., 2012; Chmielewski, 2010; Feng et al., 2008; Muley et

275 al., 2019), which has been related to increased solubility of chitosan fragments  
276 compared to high molecular weight polymer (Chmielewski, 2010). The observed  
277 increase in cell wall mass and antioxidative capacity represents a fast adaptation  
278 mechanism which may explain previous observations that microalgae are less sensitive  
279 to chronic than to acute exposure to ionizing radiation (Fuma et al., 2012). Cell wall  
280 isolates showed substantial capacity to bind heavy metals (Fig. 4):  $\text{Cu}^{2+}$ , 0.48 mg/mg;  
281  $\text{Mn}^{2+}$ , 0.38 mg/mg; and  $\text{Cr}^{3+}$ , 0.33 mg/mg of cell wall isolate (values for control  
282 samples). The irradiation of *C. sorokiniana* culture provoked a significant increase in the  
283 capacity of the cell wall to bind  $\text{Cu}^{2+}$  (Fig. 4a), whereas the capacities for  $\text{Mn}^{2+}$  and  $\text{Cr}^{3+}$   
284 binding remained unaltered (Fig. 4b, c). To the best of our knowledge, this is the first  
285 report on the metal binding capacity of isolated microalgal cell wall polymers. There are  
286 a number of reports of metal binding capacities of intact microalgal biomass (Mehta and  
287 Gaur, 2005; Wilde and Benemann, 1993), which were lower than reported in this study.  
288 For example, the capacity of *Chlorella vulgaris* biomass for  $\text{Cu}^{2+}$  binding ranged from  
289 0.01 to 0.19 mg/mg in previous studies (Mehta and Gaur, 2005). Our results are in line  
290 with reports identifying cell wall as the main biosorption component of microalgal  
291 biomass (Horikoshi et al., 1979; Klimmek et al., 2001; Mehta and Gaur, 2005; Wilde and  
292 Benemann, 1993). Further, higher affinity for  $\text{Cu}^{2+}$  than  $\text{Mn}^{2+}$  and  $\text{Cr}^{3+}$  is probably  
293 related to differences in coordinative chemistry of these metals. According to the  
294 principle of hard and soft acids and bases (HSAB),  $\text{Cu}^{2+}$  is a borderline acid, whereas  
295  $\text{Mn}^{2+}$  and  $\text{Cr}^{3+}$  are hard acids (Hancock and Martell, 1996). This means that they prefer  
296 different types of ligands/binding sites within the cell wall. The improvement of  $\text{Cu}^{2+}$   
297 binding capacity by irradiation may be important for adaptation of microalgae to extreme

298 conditions. Radiological contamination of aquatic ecosystems is usually accompanied  
299 by metal pollutants including copper, and vice versa copper mining wastewaters  
300 typically show increased levels of radiation (Dessouki et al., 2005; Fuma et al., 2012).  
301 Finally, microalgal biomass has been used as biosorbent for remediation of mining,  
302 industrial and radioactive wastewaters (Bradshaw et al., 2019; Dessouki et al., 2005;  
303 Kaplan, 2013). Our results imply that the application of microalgae in biosorbent  
304 technology could be improved by using isolated cell wall material as a sorbent rather  
305 than intact cells. However, commercial side of biomass processing has to be evaluated  
306 and taken into account.

307 All of the applied metals are paramagnetic so their coordination could be analysed using  
308 low-T EPR spectroscopy (Hagen, 2008). Fig. 5 compares EPR spectra of  $\text{Cu}^{2+}$  in the  
309 buffer, bound to cell wall isolates, and bound to chitosan.  $\text{Cu}^{2+}$  ( $S = 1/2$ ) showed EPR  
310 signal with one strong  $g$  line and four weak lines coming from hyperfine coupling with  
311  $^{63}\text{Cu}/^{65}\text{Cu}$  nuclei ( $I = 3/2$ ) along  $g_{\parallel}$ . Spectral shape and the rank order of  $g$ -values ( $g_{\parallel} >$   
312  $g > g_{\text{free electron}}$ ), imply that  $\text{Cu}^{2+}$  is in an octahedral coordination environment with  
313 tetragonal distortion in all analyzed systems (Garribba and Micera, 2006). However,  
314 hyperfine splittings ( $A_{\parallel}$ ) and  $g_{\parallel}$  values imply different nature of  $\text{Cu}^{2+}$  ligands (Peisach and  
315 Blumberg, 1974). In the buffer,  $\text{Cu}^{2+}$  spectrum showed  $g_{\parallel} = 2.31$  and  $A_{\parallel} = 14.7$  mT (Fig.  
316 5a), which are characteristic for  $\text{Cu}^{2+}$  bound to 3O and 1N ligands, and in accord with a  
317 previous study on  $\text{Cu}^{2+}$  coordination with Hepes and  $\text{OH}^{-}$  ions (Sokołowska and Bal,  
318 2005). On the other hand, the spectrum of  $\text{Cu}^{2+}$  that is bound to the isolated cell wall  
319 showed  $g_{\parallel} = 2.22$  and  $A_{\parallel} = 19.0$  mT. These values imply that  $\text{Cu}^{2+}$  is bound to 3N and  
320 1O, or to 4N ligands (Fig. 5b). This implies that glucosamine moieties represent the

321 main sites of coordinate bonding of  $\text{Cu}^{2+}$  to the cell wall. This is further substantiated by  
322 similar  $g_{\parallel}$  and  $A_{\parallel}$  values for  $\text{Cu}^{2+}$  that is bound to chitosan-like polymer, which is  
323 composed of glucosamine and N-acetylglucosamine (Fig. 5c). Our findings are in line  
324 with a previous study that identified amine, secondary amide and carboxyl groups as  
325 the most important for  $\text{Cu}^{2+}$  binding to the surface of *Spirulina* cells (Dmytryk et al.,  
326 2014). Amine groups in an electron-withdrawing system (such as glucosamine), as well  
327 as secondary amides represent borderline bases and match  $\text{Cu}^{2+}$  according to HSAB  
328 principle (Hancock and Martell, 1996). It is important to point out that spectra did not  
329 change notably for the cell wall that was isolated from irradiated algae (Fig. 5b). From  
330 these data we can speculate that the increase in  $\text{Cu}^{2+}$  binding capacity may be the  
331 result of increased accessibility to N ligands in damaged/loosened chitosan-like  
332 structure in TLS. Pertinent to this, increased binding capacity for uranium in heated  
333 biomass of *Chlorella* has been attributed to cell wall denaturation and increased  
334 accessibility of binding sites (Horikoshi et al., 1979).

335 Fig. 6a shows a characteristic six-line spectrum of  $\text{Mn}^{2+}$  ( $S = 5/2$ ,  $I = 5/2$ ) in solution.  
336 Cell wall isolates with  $\text{Mn}^{2+}$  showed similar spectra but of lower intensity (Fig. 6b), which  
337 implies that  $\text{Mn}^{2+}$  was released from the cell wall into the buffer. The remaining bound  
338  $\text{Mn}^{2+}$  did not show detectable EPR signal. The main reason for this is zero field splitting  
339 anisotropy that is promoted by the loss of rapid molecular tumbling, in combination with  
340 a large number of transitions, *i.e.* complex multiline spectrum (Ignjatović et al., 2012;  
341 Sigel and Sigel, 2000). Empirically, sufficient S/N ratio for the signal of bound  $\text{Mn}^{2+}$  is  
342 achieved when concentration of  $\text{Mn}^{2+}$  in solution is 10-50-fold lower than bound  $\text{Mn}^{2+}$   
343 (Sigel and Sigel, 2000), which was not the case here. The EPR results imply that  $\text{Mn}^{2+}$



344 is weakly bound to the cell wall and that irradiation did not alter  $Mn^{2+}$  binding capacity, in  
345 accordance with the biochemical measurements. Next,  $Cr^{3+}$  ( $S = 3/2$ ) has two nuclear  
346 spins:  $I = 0$  (natural abundance of 83%), and  $I = 3/2$  (9.5%) (Azamat et al., 2013). This  
347 results in an EPR spectrum containing one strong line for the first isotope, and four  
348 weak lines for the latter (Fig. 6c). Similarly to  $Mn^{2+}$ , the binding of  $Cr^{3+}$  to large slowly-  
349 tumbling molecules results in significant line broadening due to zero field splitting  
350 anisotropy and fast relaxation (Andriessen and Groenewege, 1976; Hagen, 2008). Line  
351 broadening could be also related to paramagnetic effects, *i.e.* dipolar interactions  
352 between  $Cr^{3+}$  ions that are closely positioned within cell wall matrix (Hagen, 2008;  
353 Padlyak et al., 2000). Therefore, no resolved EPR spectrum was obtained for  $Cr^{3+}$  that  
354 was bound to cell wall (Fig. 6d). The lack of signal of  $Cr^{3+}$  in solution means that  $Cr^{3+}$  is  
355 more strongly bound to the cell wall than  $Mn^{2+}$ . This may be related to stronger  
356 electrostatic interactions with negative charges on the cell wall for more positive  $Cr^{3+}$ .

#### 357 **4. Conclusions**

358 In summary, our results demonstrate that the microalgal cell wall is a dynamic and  
359 stress-responsive structure that is involved in fast adaptation to environmental  
360 challenges. Within a day after exposure to ionizing radiation, *C. sorokiniana*  
361 strengthened its first line of defence against the external environment. The wall became  
362 thicker and showed altered composition with increased fraction of uronic acids in the  
363 fibrillar layer. The cell wall showed improved capacity to remove the main reactive  
364 product of water radiolysis. In addition, the isolated microalgal cell wall exhibited high  
365 copper binding capacity, which was further increased by irradiation. These fast  
366 adaptation mechanisms are most likely part of a complex process of responses to

367 different stressors. Knowledge of these responses is essential for the understanding of  
368 the ecotoxicology of ionizing radiation and the application of microalgae in metal  
369 remediation, wastewater treatment, and biosorbent industry.

370

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374

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539

540 FIGURE CAPTIONS

541 **Fig. 1.** Representative TEM micrographs of *C. sorokiniana* cells and the analysis of cell  
542 wall parameters. (a) Characteristic TEM micrographs – controls and cells exposed to  
543 different doses of X-ray irradiation in the stationary phase. Analysis was performed one  
544 day after the treatment. Cell wall structure shows two main layers: trilaminar sheath –  
545 TLS (1) and fibrillar wall (2), see the enlarged area of the cell (dashed line box). (b) Data  
546 on thickness of cell wall layers; (c) The yield of cell wall (mass per g of fresh weight  
547 (FW). Mean control values (Ctrl) are presented as full line  $\pm$  SE (dashed line). Statistical  
548 significance - \*  $p < 0.05$ .

549 **Fig. 2.** FTIR spectra of cell wall isolates from a *C. sorokiniana* stationary phase culture  
550 that was untreated (control) or irradiated (2 Gy, similar spectra were recorded for 5 Gy).  
551 Circles mark the areas of interest.

552 **Fig. 3.** EPR spectra of DEPMPO spin-adduct with  $\text{HO}^\cdot$  that are produced in the absence  
553 or the presence of cell wall isolates. (a) Fenton reaction:  $\text{Fe}^{2+}$  (1 mM) +  $\text{H}_2\text{O}_2$  (3 mM).  
554 Full circle - the amplitude of this peak was used to calculate antioxidative capacity. (b)  
555 Cell wall of untreated algae (controls); (c) Cell wall of algae exposed to 1 Gy; (d) Cell  
556 wall of algae exposed to 2 Gy; (e) Cell wall of algae exposed to 5 Gy. Antioxidative  
557 capacity is presented as mean  $\pm$  SE. \* - statistically significant compared to Fenton  
558 reaction ( $p < 0.05$ ).

559 **Fig. 4.** The capacity of *C. sorokiniana* cell wall isolates to bind metal ions. (a)  $\text{Cu}^{2+}$   
560 binding capacity of cell wall polymers from untreated and irradiated microalgae. (b)  $\text{Mn}^{2+}$   
561 binding capacity. (c)  $\text{Cr}^{3+}$  binding capacity. Results are presented as means  $\pm$  SE. Mean

562 control values (Ctrl) are presented as full line  $\pm$  SE (dashed line). \* - statistical  
563 significance compared to non-irradiated culture ( $p < 0.05$ ).

564 **Fig. 5.** 77 K EPR spectra of  $\text{Cu}^{2+}$ . (a)  $\text{Cu}^{2+}$  (1.5 mM) in Hepes buffer (20 mM; pH 7.5);  
565 (b)  $\text{Cu}^{2+}$  + cell wall isolates from control and irradiated microalgae in the buffer. (c)  $\text{Cu}^{2+}$   
566 + chitosan. Recording parameters were: power attenuation, 25 dB; modulation  
567 amplitude, 0.8 mT; number of scans/accumulations, 10. Gray lines – simulations that  
568 delivered the presented  $g$  and  $A$  values.

569 **Fig. 6.** 77 K EPR spectra of  $\text{Mn}^{2+}$  and  $\text{Cr}^{3+}$ . (a)  $\text{Mn}^{2+}$  (1.5 mM) in Hepes buffer (20 mM;  
570 pH 7.5); (b) Cell wall isolates (from control and irradiated microalgae) that were  
571 incubated with  $\text{Mn}^{2+}$  and placed into the buffer. (c)  $\text{Cr}^{3+}$  (1.5 mM) in Hepes buffer (20  
572 mM; pH 7.5). Arrowheads mark 4 lines of  $\text{Cr}^{3+}$  with  $I = 3/2$ . (d) Cell wall isolates (from  
573 control and irradiated microalgae) that were incubated with  $\text{Cr}^{3+}$  and placed into the  
574 buffer. Recording parameters were: power attenuation, 25 dB; modulation amplitude,  
575 0.8 mT; number of scans, 10.

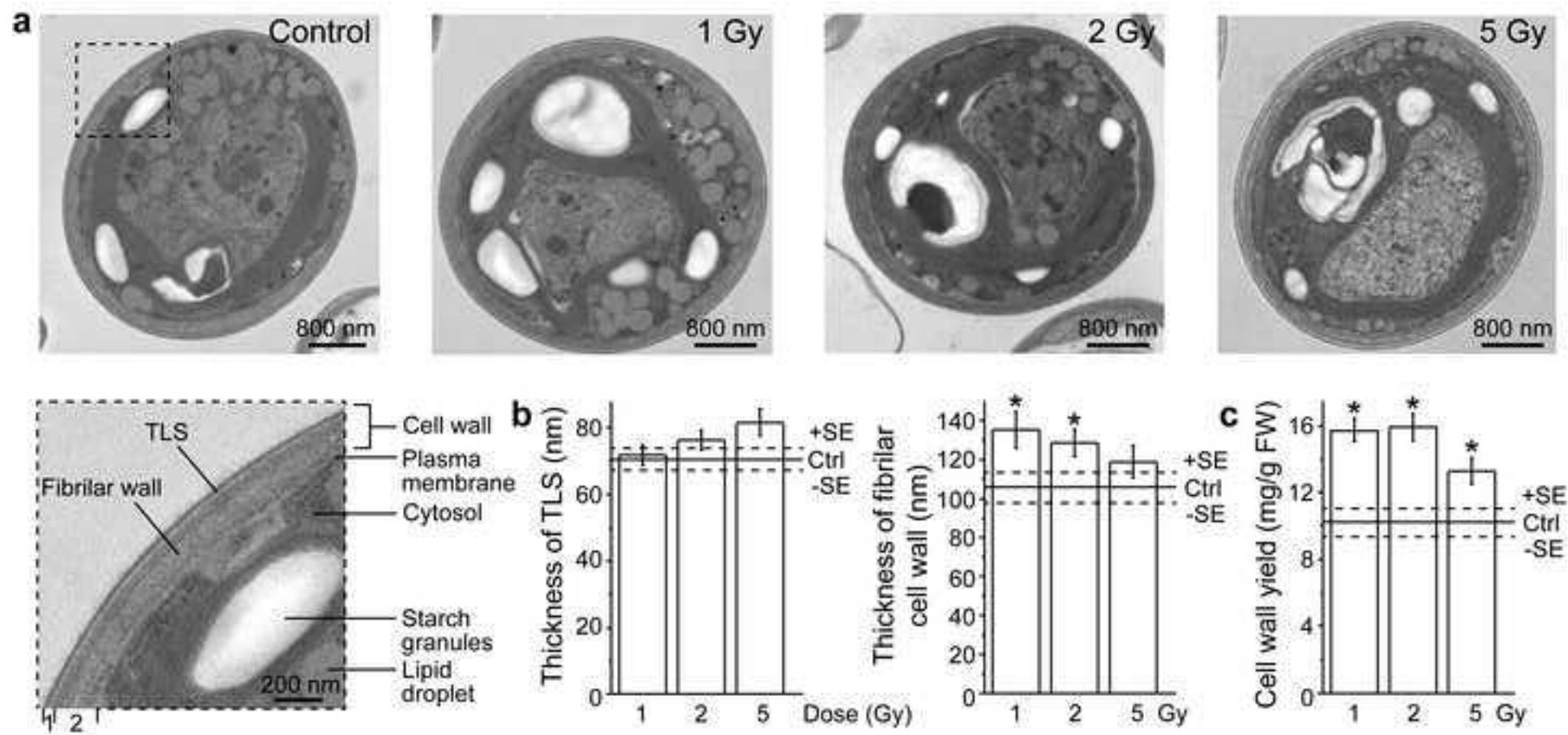


Figure 2

